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[COMMUNICATION]

Properties of (Na^+, K^+)-ATPase in the Canine Kidney Cell Line (MDCK) and its Ouabain-Resistant Mutant

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ABSTRACT—The properties of (Na^+, K^+)-ATPase from the Madin-Darby canine kidney cell line (MDCK) and its ouabain-resistant mutant (MDCK-OR) were compared using a microsomal fraction treated with sodium deoxycholate. In MDCK-OR, the activity of ouabain-insensitive Mg^{2+}-ATPase increased, while that of (Na^+, K^+)-ATPase was less than that of wild-type MDCK. The parameters for Na^+, Mg^{2+} and ATP were essentially the same in the two cell strains. However, the apparent Km of (Na^+, K^+)-ATPase for K^+ was remarkably lower than that of MDCK. The ouabain-inhibition of the enzyme in MDCK was competitive to K^+ with an apparent Ki of 20 nM. The half-maximal inhibition of the MDCK-OR cell enzyme by ouabain was noted at 2–5 μM, independent of the concentration of KCl. When the enzyme was preincubated with Na^+, Mg^{2+}, ATP and various concentrations of ouabain, biphasic curves were obtained for enzyme inhibition. The enzyme activity low in sensitivity toward ouabain was found to be about 15% the total activity in MDCK but about 60% in MDCK-OR.

INTRODUCTION

The Madin-Darby canine kidney cell (MDCK) has been used as a model system for the study of renal transport *in vitro*, since MDCK retains some features of renal tubular epithelia [1, 2]. MDCK is sensitive to vasopressin but not to parathyroid hormone or calcitonin [3, 4], and can also synthesize characteristic glycosphingolipids of dog kidney [3, 5–7]. Na^+ pumps have been demonstrated in MDCK by assay of (Na^+, K^+)-ATPase activity (EC 3.6.1.3) [8, 10] and ouabain binding [2, 11]. Previously, we isolated an ouabain-resistant mutant (MDCK-OR) derived from MDCK [12] and noted remarkable changes in the glycolipid composition of the mutant [6, 7], thus indicating Na^+ pump activity to possibly be modulated by membrane glycolipids. Although several ouabain-resistant mammalian cells have been reported from other laboratories [13–18] and in two of these strains two classes of ouabain binding sites were found to be present [14, 19], the features of the enzyme activity have yet to be clarified in detail. In the present study, various properties of (Na^+, K^+)-ATPase in wild-type MDCK and MDCK-OR were compared and the ouabain-resistant enzyme in MDCK-OR was found to be more sensitive toward K^+ ion than the ouabain-sensitive enzyme in wild-type MDCK.

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MATERIALS AND METHODS

Cells

MDCK cells and an ouabain-resistant clone (MDCK-OR) were cultured as described previously [3, 7, 12]. After being washed with phosphate-buffered saline (PBS), the cell monolayer at confluence was harvested with a rubber policeman. The packed cells were washed with PBS and with 1 mM EDTA in 0.25 M sucrose solution adjusted to pH 7.4 by tris(hydroxymethyl)aminomethane (Tris). It was then stored at −80°C until use.

Preparation of the microsomal fraction

The method of Robbins and Baker was modified for preparation of deoxycholate-treated microsomes [14]. About 500 mg wet weight of cells were suspended in 1.2 ml of 1 mM EDTA solution (pH 7.4), followed by gentle stirring on ice for 20 min. To this suspension 120 μl of 5% sodium deoxycholate (Sigma) in 1 mM EDTA solution were added (final concentration, 0.34%) and stirred for 20 min. This solution was diluted with 7.2 ml of EDTA solution and centrifuged at 10,000$\times g$ for 15 min. The pellet thus obtained was treated again with sodium deoxycholate as mentioned above and centrifuged again to obtain a pellet (MT). The first and second supernatants were combined and centrifuged at 156,000$\times g$ for 2 hr at 4°C, and the supernatant (Sup) was removed. The residual pellet (MS) was resuspended in EDTA solution at a concentration exceeding 2 mg protein per ml and stored on ice. The (Na^+, K^+)-ATPase activity of this fraction was stable for more than 3 weeks.

Assay of ATPase activity

The standard assay mixture for total ATPase activity contained 30 mM histidine (pH 7.6), 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 3 mM ATP(Tris salt) (Sigma) and an enzyme preparation (10–100 μg protein) in 0.5 ml. For assay of ouabain-insensitive ATPase activity, KCl was omitted from the standard assay mixture and 0.5 mM ouabain was added. The non-specific hydrolysis of ATP was assayed using the heat-inactivated enzyme. (Na^+, K^+)-ATPase activity was estimated by subtracting the ouabain-insensitive activity from total ATPase. Mg^{2+}-ATPase activity was estimated by subtracting non-specific ATP hydolyzing activity from ouabain-insensitive ATPase activity. The assay was performed at 37°C with shaking and terminated by the addition of 0.5 ml of a cold molybdate solution. The molybdate solution was prepared by dissolving freshly $FeSO_4 \cdot 7H_2O$ (736 mg) in 10 ml of 1.15 N H_2SO_4 containing 1% ammonium molybdate. The reaction mixture was stirred, incubated for 3 min at 37°C and cooled on ice. The solution was centrifuged at 1,000$\times g$ for 7 min, and the inorganic phosphate in the supernatant was measured colorimetrically at 750 nm. The ouabain-sensitive ATPase activity was proportional to incubation time between 0 and 35 min and the amount of protein between 0 and 100 μg. Protein content was determined by the procedure of Lowry *et al.* using bovine serum albumin as the standard [20].

RESULTS

ATPase activity in wild-type MDCK cells and the ouabain-resistant mutant

The activity of ouabain sensitive (Na^+, K^+)-ATPase and Mg^{2+}-ATPase of MDCK and MDCK-OR cells was determined in cell homogenates and in three subcellular fractions in triplicates. Microsomal fractions from both cells treated with sodium deoxycholate each showed (Na^+, K^+)-ATPase activity to be more than twice that of the homogenates, and total activity was recovered by more than 80% in each of these fractions as shown in Table 1. The MT and Sup fractions of both cells showed very low activity. The distribution of the activity in these subcellular fractions of the two cell types was the same. It is of interest that the specific activity of (Na^+, K^+)-ATPase in the microsome (MS) of MDCK-OR was only 57% the corresponding activity of MDCK (significance level $P<0.001$). In contrast, ouabain-insensitive Mg^{2+}-ATPase activity in MS of MDCK-OR increased to as much as one and a half times that of MDCK. Specific enzyme activity in the other subcellular fractions and cell homogenates was essentially the same in MDCK and MDCK-OR. The kinetic parameters of microsomal (Na^+, K^+)-ATPase for ligands (Na^+, K^+, Mg^{2+} and ATP) were determined from Lineweaver-Burk plots. The apparent Km values for K^+ of the MDCK and MDCK-OR enzymes were 1.7 and 0.5 mM, respectively (significance level $P<0.02$, Table 2). The apparent Km values of the MDCK-OR enzyme for Na^+, Mg^{2+} and ATP did not differ from those of MDCK enzyme, as evident from Table 2.

Enzyme sensitivity toward ouabain inhibition

The inhibition of MDCK (Na^+, K^+)-ATPase by ouabain was assessed at various concentrations of KCl (1, 3 and 10 mM) (Fig. 1A) and was found to

TABLE 1. Distribution of (Na^+, K^+)-ATPase and Mg^{2+}-ATPase activity in subcellular fractions

Fraction	Protein	(Na^+, K^+)-ATPase		Mg^{2+}-ATPase	
	(%)	specific activity (nmol/mg/min)	total activity (%)	specific activity (nmol/mg/min)	total activity (%)
MDCK cell					
Homogenate		61.8±7.8		26.6± 3.1	
MT	18.8±1.3	10.4±3.8	3.8±1.7	58.8±17.3	36.0±3.1
MS	33.8±1.1	139.8±5.9[a]	86.8±1.7	48.3± 5.9[c]	55.0±2.4
Sup	47.4±1.6	10.8±1.0[b]	9.5±1.2	5.4± 0.4	9.0±2.2
MDCK-OR cell					
Homogenate		35.1±7.0		28.5± 3.7	
MT	18.3±1.2	14.9±9.7	8.6±4.7	57.0± 6.7	28.8±3.5
MS	29.3±1.2	80.3±6.6[a]	79.9±6.1	74.3± 7.4[c]	60.1±4.5
Sup	52.4±2.3	6.4±0.8[b]	11.4±1.3	7.6± 0.6	11.1±2.1

Each value (mean±S.D.) was obtained from three separate experiments.
Significance level by *t*-test: [a], $P<0.001$; [b], $P<0.01$; [c], $P<0.02$.
MT, 10,000×*g* pellet; MS, 10,000−156,000×*g* pellet; Sup, 156,000×*g* supernatant.

TABLE 2. Kinetic parameters of (Na^+, K^+)-ATPase in MDCK and MDCK-OR cells

Apparent Km	MDCK	MDCK-OR
Na^+ (at 10 mM K^+)	10.2 ±0.6 mM (3)	9.7 ±0.5 mM (3)
K^+ (at 100 mM Na^+)	1.7 ±0.2 mM (4)[a]	0.5 ±0.1 mM (4)[a]
Mg^{2+}	0.54±0.09 mM (3)	0.65±0.12 mM (3)
ATP	0.82±0.22 mM (3)	0.71±0.24 mM (3)
Ki for ouabain	20 nM	(2–5 μM)[b]

Apparent Km values were obtained from Lineweaver-Burk plots.
Standard assay conditions were used. Each value is the mean±S.D. of the experiments. Figure in parenthesis indicates the number of experiments.
Significance level by *t*-test: [a], $P<0.02$
[b] Half-maximal inhibition was obtained from FIG. 1B.

depend on the concentration of KCl. The lower the concentration of KCl, the lower was the concentration of ouabain for half-maximal inhibition of the enzyme. When inhibition curves were drawn in a Dixon plot (data not shown), the lines converged at a point representing a Ki value of 20 nM. Too, double reciprocal plots for various concentrations of KCl in the presence or absence of ouabain also converged on a point at the ordinate (data not shown). It would thus appear that inhibition by ouabain is in competition with K^+ ions. The ouabain inhibition curves of MDCK-OR enzyme showed no dependence on KCl concentration (Fig. 1B). The ouabain concentration for half-maximal inhibition of the MDCK-OR enzyme was 2–5 μM (Table 2).

Effect of preincubation on ouabain inhibition

Following the preincubation of the MS fraction with Na^+, Mg^{2+}, ATP and ouabain at various concentrations, the remaining activity of (Na^+, K^+)-ATPase was measured. The MS fraction of MDCK, preincubated for 10 or 20 min with ouabain, was more sensitive to ouabain than without preincubation (Fig. 2A). The ouabain high-sensitive enzyme activity was about 85% the total activity, the remaining being low at a concentration of ouabain exceeding 10^{-7} M.

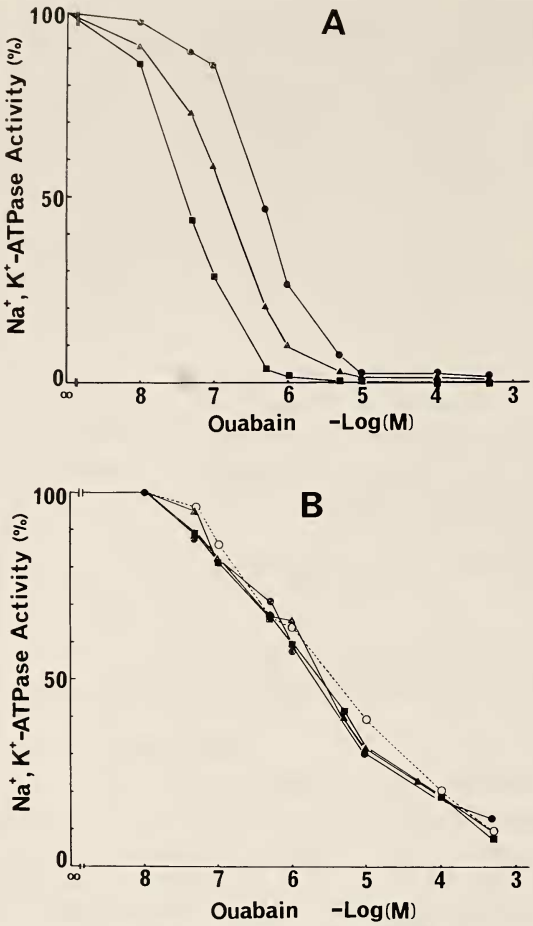

FIG. 1. Effect of KCl on the inhibition of (Na^+, K^+)-ATPase by ouabain. The enzyme assay was performed in the standard mixture or a mixture modified for KCl concentration in the presence of the indicated concentrations of ouabain. The ratio of residual activity to total activity is shown. (A), MDCK: 10 mM KCl (●); 3 mM KCl (▲); 1 mM KCl (■). (B) MDCK-OR: 10 mM KCl (○); 3 mM KCl (▲); 2 mM KCl (■); 1 mM KCl (●).

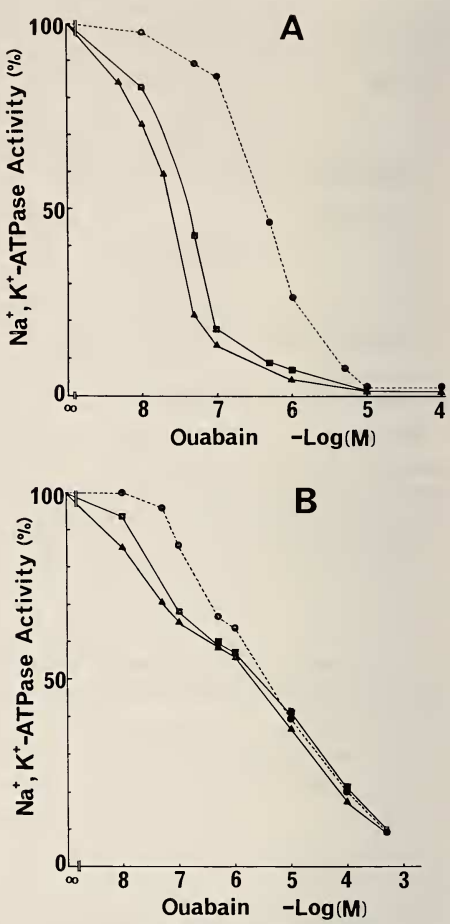

FIG. 2. Effect of preincubation with ouabain on the inhibition of (Na^+, K^+)-ATPase activity of MDCK (A) and MDCK-OR (B). Media containing 30 mM histidine, 100 mM NaCl, 5 mM $MgCl_2$, 3 mM ATP and microsomal enzyme were preincubated with ouabain at the indicated concentrations at 37°C for 10 min (■) or 20 min (▲). Reaction was initiated by the addition of 10 mM KCl. Activity was measured as described in Materials and Methods. Data without preincubation (●) were taken from FIG. 1.

The enzyme of MDCK-OR also gave biphasic curves for ouabain inhibition when preincubated with ouabain for 10 or 20 min (Fig. 2B). At concentrations of ouabain above 10^{-6} M, inhibition was independent of preincubation time. The above low level of sensitivity increased to about 60% the total activity in MDCK-OR. At ouabain concentrations below 10^{-7} M, inhibition was observed to depend on preincubation, as noted for the high-sensitive enzyme of MDCK. When the MS fraction of both cells was preincubated with 10^{-7} M ouabain, high-sensitive activity was inhibited to 0% the original activity within 10 min, while inhibition of the low-sensitive enzyme showed no change during preincubation of more than 10 min (Fig. 3). The low-sensitive enzyme of MDCK-OR was also confirmed to be inhibited at high ouabain concentration (above 10^{-5} M) re-

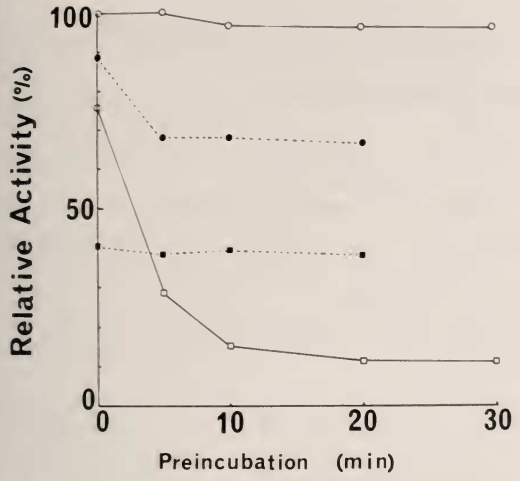

FIG. 3. Time course of preincubation for the inhibition of the enzyme by ouabain. The enzyme of MDCK or MDCK-OR cells was preincubated with ouabain for the indicated times, and then assayed as described in FIG. 2. MDCK cell microsomes without ouabain (○), MDCK cell microsomes with 10^{-7} M ouabain (□), MDCK-OR cell microsomes with 10^{-7} M ouabain (●) and MDCK-OR cell microsomes with 10^{-5} M ouabain (■).

gardless of the period of preincubation.

DISCUSSION

From the results presented above, it is evident that the specific activity of (Na^+, K^+)-ATPase of MDCK-OR is lower than that of MDCK. This quite consistent with the reduction in ouabain binding in the ouabain-resistant strains of MDCK, as reported by Soderberg *et al.* [19]. It is thus quite likely that the resistance of MDCK-OR to the cytotoxic effect of ouabain is due to the lower sensitivity of (Na^+, K^+)-ATPase toward the inhibitor. We previously found the (Na^+, K^+)-ATPase activity in an ouabain-resistant mouse cell line (JLS-V_9OR) to be similar to that of wild-type cells [16]. The wild-type HeLa cell and its mutants have also been shown identical in enzyme activity [14]. However, enzyme activity corresponding to equal amounts of DNA (or cell number) was noted essentially the same in both MDCK and MDCK-OR cells, the ratio of protein to DNA(w/w) in MDCK and MDCK-OR being 30.4 and 55.5, respectively [7]. There has never been any indication that ouabain-insensitive Mg^{2+}-ATPase activity is higher in a mutant cell than in a wild-type cell.

The MDCK enzyme showed ouabain-inhibition of a competitive type with K^+ ions, while the inhibition of (Na^+, K^+)-ATPase of MDCK-OR by ouabain was not affected by K^+ ion concentration. The apparent Km value for the MDCK-OR enzyme differed from that of the MDCK enzyme only for K^+ ions. Although a high Km value for the K^+ ions of the ouabain-resistant cell enzyme has been reported [14], a low Km value for these ions has never been indicated for ouabain-resistant cells. The low Km value for K^+ in the MDCK-OR enzyme indicates the ouabain-resistant enzyme of MDCK-OR to possibly be more sensitive to K^+ ions than the ouabain-sensitive enzyme of MDCK. The two different enzyme activities for the ouabain-resistant HeLa cells [14] were also demonstrated in MDCK-OR and even in MDCK. The two classes of (Na^+, K^+)-ATPase could be distinguished by their sensitivity to ouabain when the phosphorylated intermediate of the enzyme was preincubated with ouabain in the absence of K^+ ions [21]. Since the proportion of low-sensitive activity is considered the same as that of high-sensitive activity in the ouabain-resistant enzyme [14, 19], it appears strange that the proportion of the low-sensitive enzyme activity was 15% in MDCK and 60% in MDCK-OR.

The ouabain-resistance of (Na^+, K^+)-ATPase has been ascribed to the primary structure of the α subunit of (Na^+, K^+)-ATPase [22]. However, the microenvironment of the enzyme in the plasma membrane may also be important for the function of the enzyme. Galactosyl sulfatide, a sulfated glycosphingolipid, may possibly be a cofactor of (Na^+, K^+)-ATPase [23–25]. Previously we reported changes in certain lipid components in MDCK-OR [6, 7] and the galactosyl sulfatide content of MDCK-OR to be 2.7 times that of MDCK [7]. Sulfatide thus appears to affect the ouabain binding site or K^+ binding site of (Na^+, K^+)-ATPase.

EDTA-solubilized proteins which regulate ouabain-sensitivity have also been reported [26]. Since in this study, a buffer containing EDTA was used for preparation of the microsomes, there

would of course not be the possibility that these proteins regulate ouabain-sensitivity in MDCK and mutant cells [27].

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